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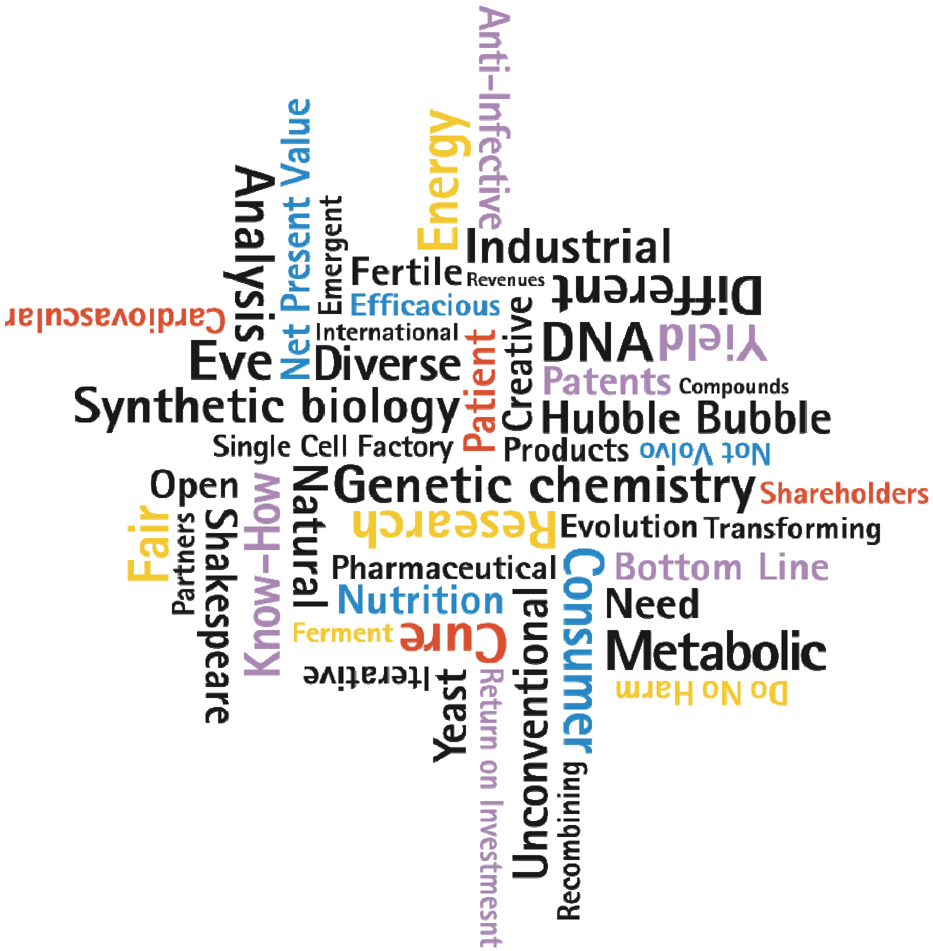
In-cell Enzymatic Glycosylation: A Way to Improve Productivity of Heterologous Biosynthesis Pathways in Micro-Organism

Esben Hansen
Evolva



In-cell enzymatic glycosylation

A way to improve productivity of heterologous biosynthesis pathways in micro-organisms

Biarritz June 7th 2012

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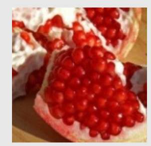
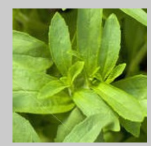
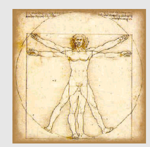
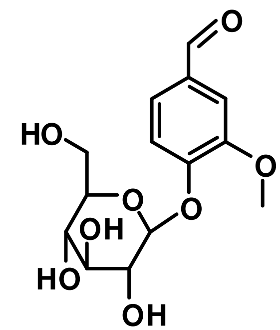
Evolva Snapshot

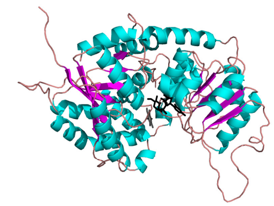
- **Unique, proven, widely applicable, biosynthesis technology**
  - Creates novel products and production methods in yeast
  - More than 50 patent families filed to date
- **Product focus in 3 ”health & wellness” areas**
  - Natural flavorants and sweeteners: Vanilla, Stevia
  - Protection against microbial pathogens: Pomecins
  - Diabetes & obesity: EV-077, Stevia
- **B2B model focused on core competencies**
  - Provide innovative ingredients to other companies
  - Partnerships with Roche, IFF, BASF, Roquette, US DoD
  - 2010 revenues CHF 18.6 mn (1H 2011 CHF 6.9mn)
- **Many pharma & food majors want to grow in space**
  - But have limited partnering or acquisition options
  - Evolva is well positioned with differentiated assets and a highly experienced team

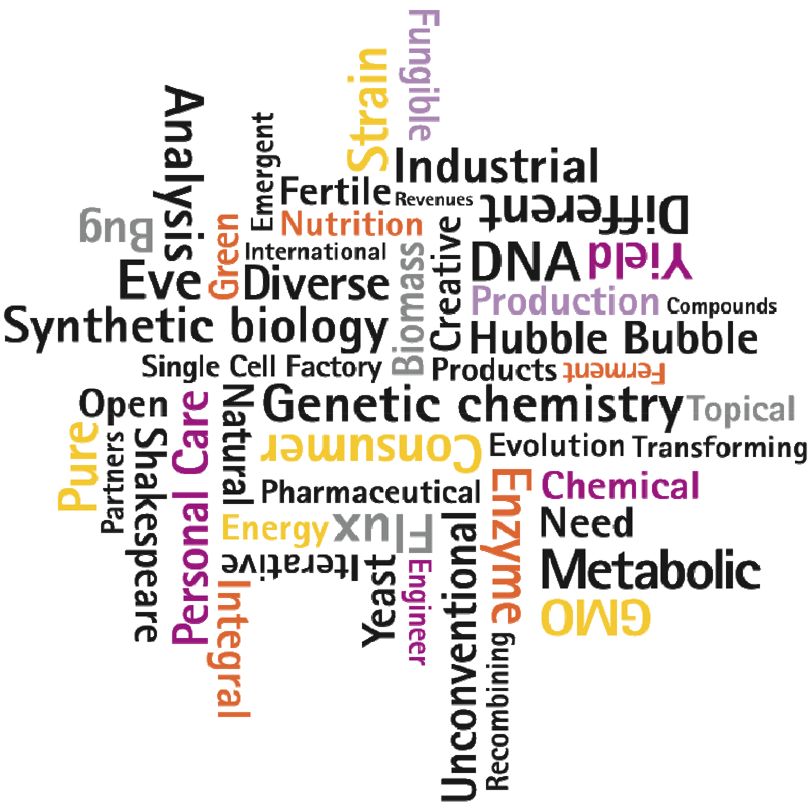
	Product Family	On Market	Addressa Marke
	Vanilla	2014	$ 0.4 b
	Pomecin	2015	$ 2 b
	Stevia	2015	$ 4 b
	EV-077	2017	$ 10 b

This Presentation

- **Glycosylation and the effects of glycosylation**
- **Glycosyltransferases**
- **Examples**
  - Vanillin biosynthesis in yeast
  - Saffron biosynthesis in yeast
  - Stevia biosynthesis in yeast







Glycosylation

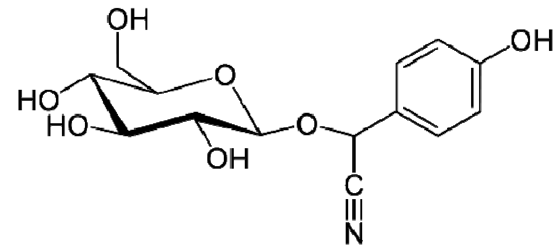
Glycosylation

- **A glycoside is a molecule in which a sugar group is bonded through its anomeric carbon to another carbon group via an O-glycosidic, S-glycosidic, or N-glycosidic bond**

- **"Small molecule" glycosides play important roles in especially micro-organisms and plants, e.g. in defence**
  - Some toxic defence compounds are stored as non-toxic glycosides
    - E.g. dhurrin, vanillin-glucoside and glucosinolates

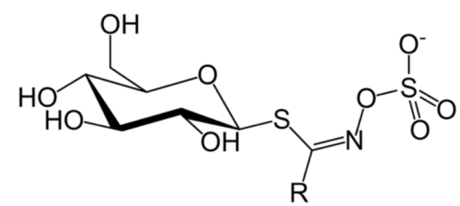
- **In animals, small compound glycosylation is used as a mean for detoxifying and excreting unwanted lipophilic compounds**
  - Glycosylation is performed by promiscuous glucuronosyltransferases in the liver
  - Water solubility is increased, facilitating excretion via the kidneys

- **Typical effects of glycosylating a scaffold**
  - Decreases the compound's cellular toxicity
  - Increases the compound's aqueous solubility
  - Increases the compound's biosynthesis rate and yield
  - In some cases: Stabilization of the product molecule



Dhurrin

Cyanogenic glucoside
Aglycon is unstable and degradation results in release of cyanide



Glucosinolate

Hydrolyzed by myrosinases upo
tissue damage, resulting in release of biologically active compounds

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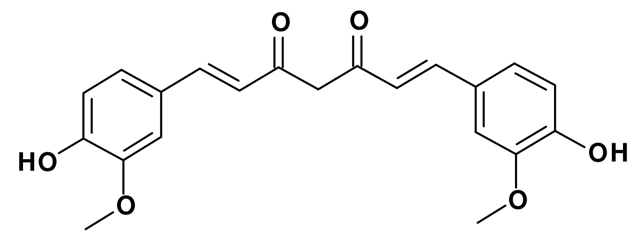
Glycosylation Increases Solubility of Curcumin

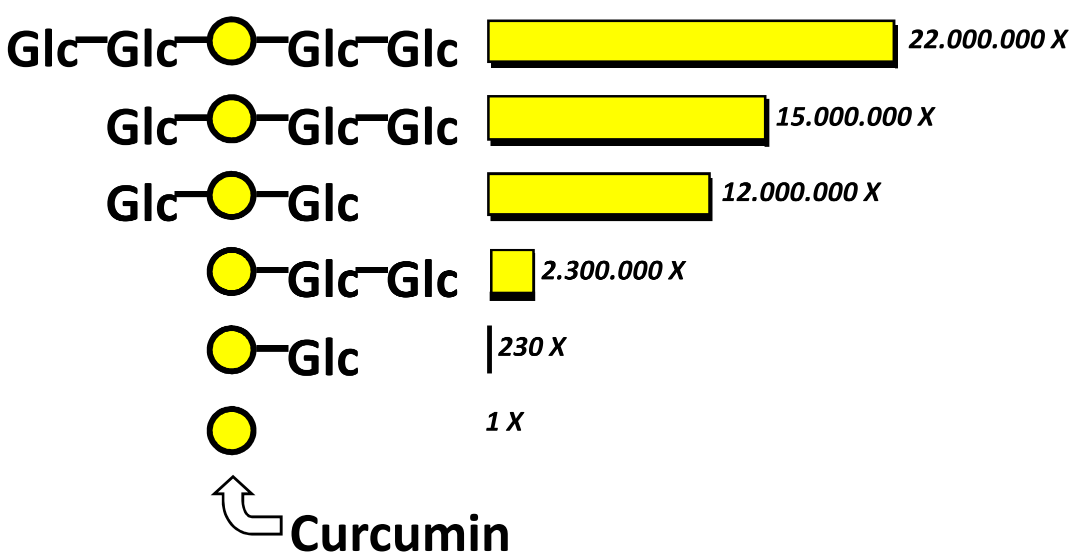
Principal curcuminoid of the popular Indian curry spice turmeric

***Increased solubility**: Insoluble curcumin gains solubility by glucosylation (Kaminaga* et al.*)*

Curcumin



Solubility increase

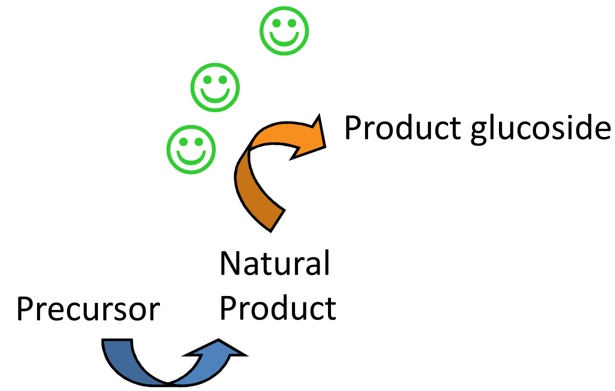


Use:

Several experimental (cancer, anti-inflammation, etc.)

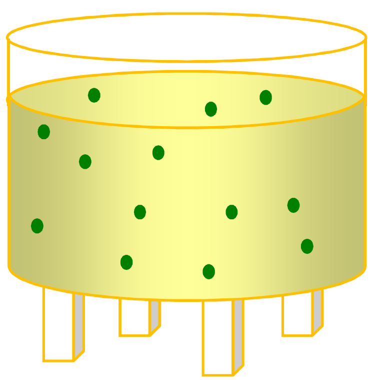
Ref.: Kaminaga *et al.* (2003) FEBS Lett. 555: 311

Amplification of Microbial Production of Toxic Compounds

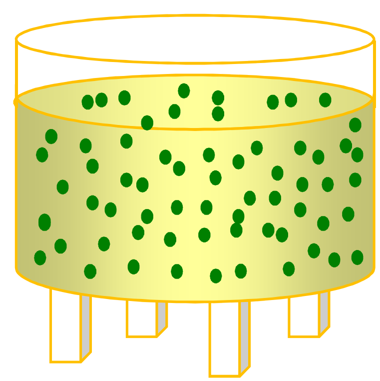


Production microorganism **dies** when high concentration of toxic compound is formed

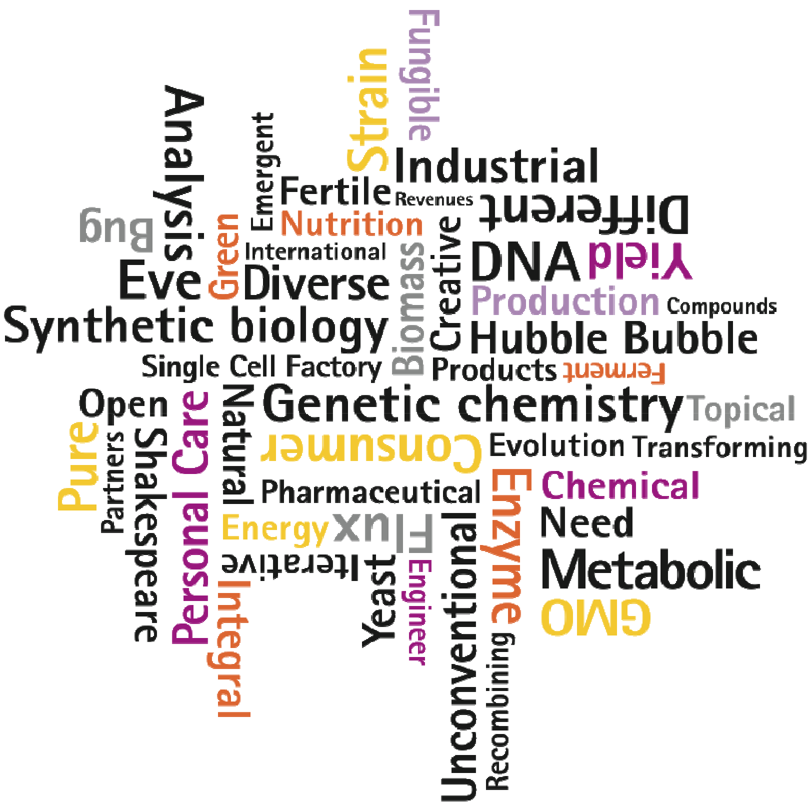
Production microorganism **survives** when toxic compound formed is **glycosylated**



Toxic aglycon



Less toxic glycoside



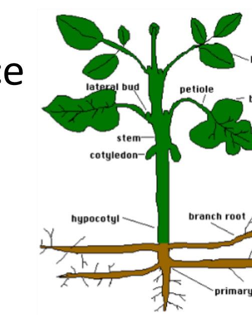
Glycosyltransferases

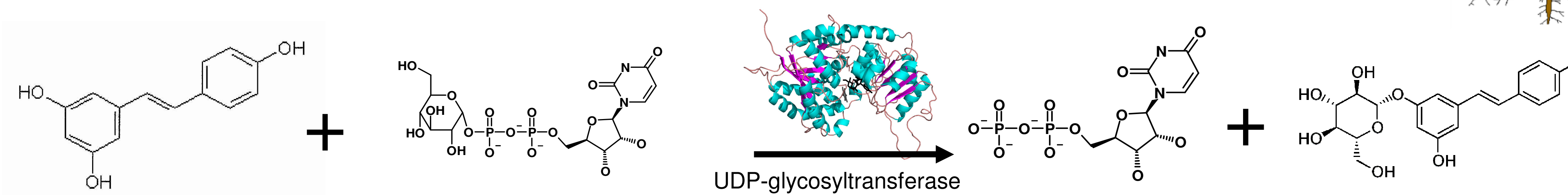
“Family 1”

Family 1 Glycosyltransferases – The UGTs

- A GT is an enzyme that can transfer a sugar from one molecule to another

- Family 1 UDP-glycosyltransferases (UGTs)
  - 1 out of 91 GT families – a large family present in both animals, plants and microorganisms
  - Small hydrophobic molecules as natural substrates
  - Involved in synthesis of many different secondary plant metabolites: Pigments, defence compounds, flavour and more
  - Involved in detoxification of xenobiotic compounds



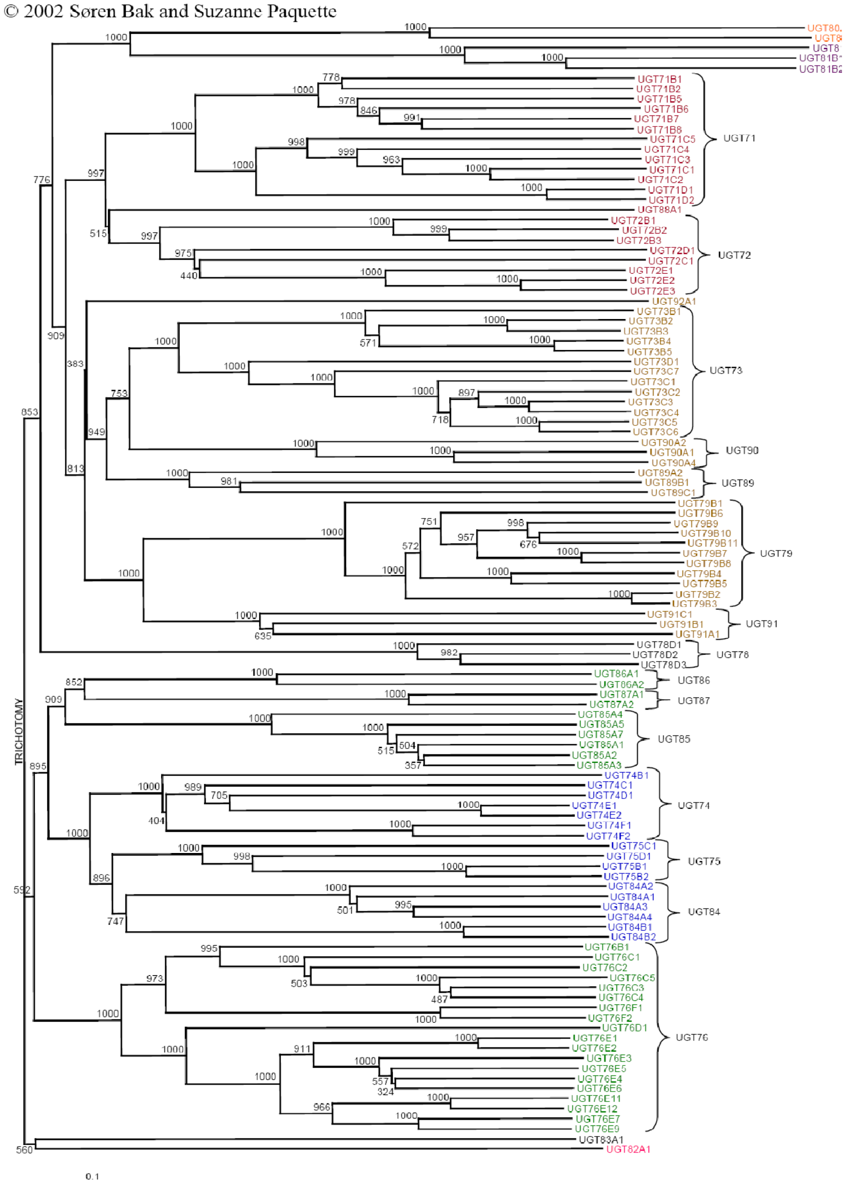


Resveratrol + UDP-glucose → UDP + Resveratrol-β-glucoside (Piceid)

  - Use uridine diphosphate-activated sugars as sugar donors
  - The catalytic mechanism is inverting, resulting in a β-configuration of the glycosidic linkage

Family 1 UGTs

© 2002 Søren Bak and Suzanne Paquette



- Most uses UDP-glucose as sugar donor
- Many plant UGTs have shown to be very promiscuous towards acceptor substrate
- *Arabidopsis thaliana* contains 122 different UGTs
- 70% of 87 small molecule drugs were glucosylated (Kristensen et al., 2008)

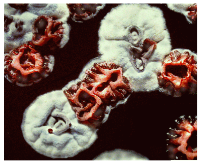
Picture: Universität Karlsruhe
Botanisches Institut

ooted phylogenetic tree – *Arabidopsis thaliana* UGTs

Evolva’s Glycosylation Platform


Arabidopsis thaliana


Stevia rebaudiana


Gram+ bacteria


Drosophila melanogaster


Sorghum bicolor


Catharanthus roseus


Neisseria meningitidis

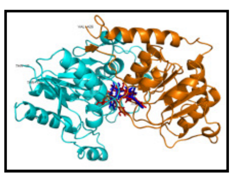

Bos taurus


Vanilla planifolia


Crocus sativus

Present platform: 220 GT enzymes

Expressed in *E.coli, P. pastoris, S. cerevisiae* and/or *in vitro*


Chimeric UGTs


Citrus maxima

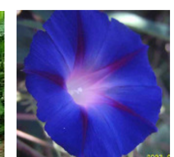

Bellis perennis

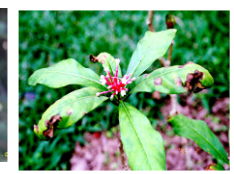

Lycopersicon esculentum


Nicotiana tabacum


Petunia hybrida

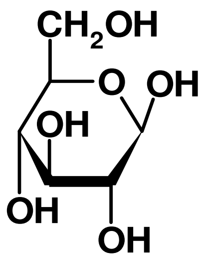

Phaseolus vulgaris


Ipomoea sp.

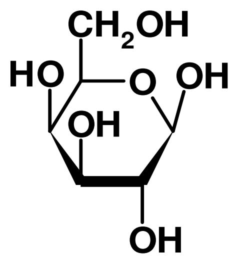

Rauwolfia serpentina


Oryza sativa

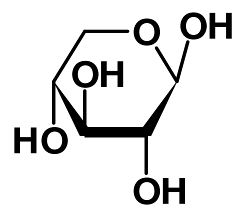
Possible Carbohydrate Species



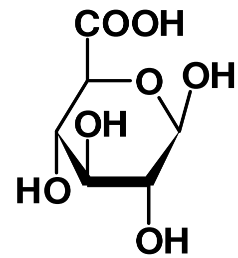
D-Glucose



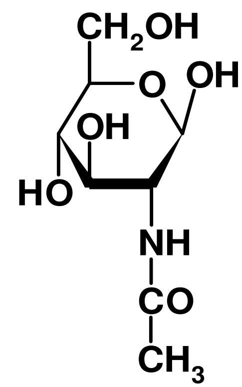
D-Galactose



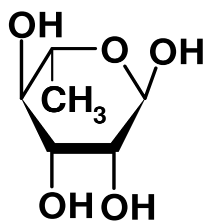
D-Xylose



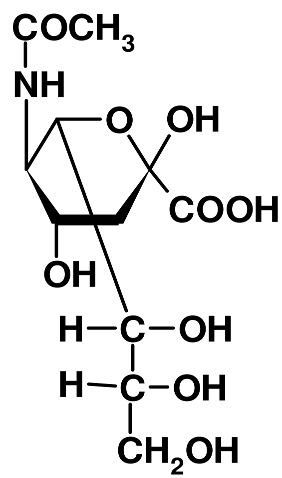
D-Glucuronic acid



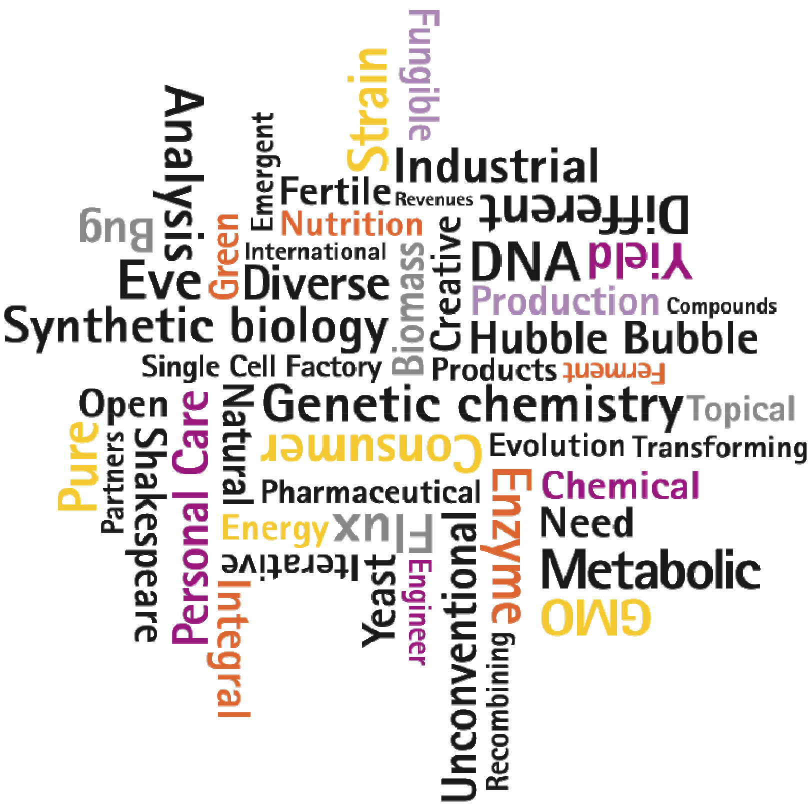
N-Acetyl-glucosamine



L-Rhamnose



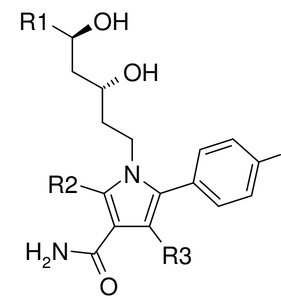
N-Acetyl-neuraminic acid
(one type of sialic acid)



In-cell enzymatic glycosylation

The Process of Making an In Cell Glycosylation for a Biosynthetic Process

- **1. Prerequisites:**
  - Product needs to have a suitable hydroxy, thio or amino group that can be glycosylated
  - The added sugar moiety is to some extent “wasted” – product therefore needs to be a high or medium priced product



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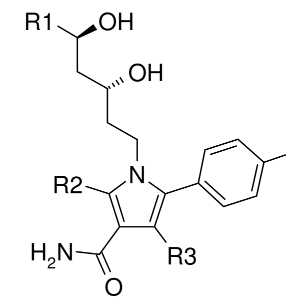
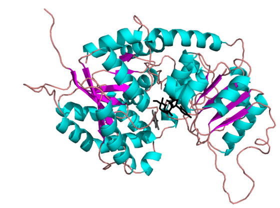
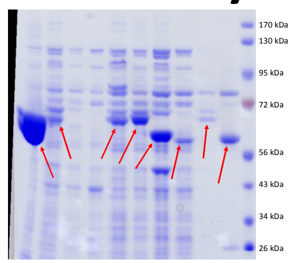


- **2. Finding a suitable glycosyltransferase that can perform the desired glycosylation**

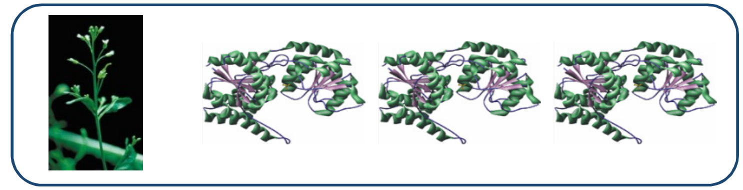


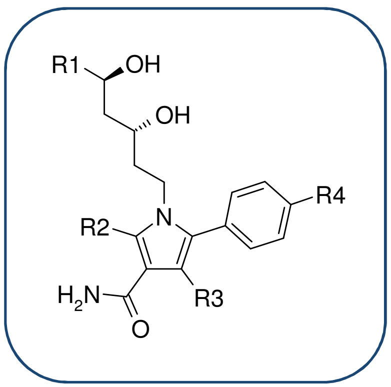
UGT Screening Assay - Finding a Suitable UGT

Purified enzyme

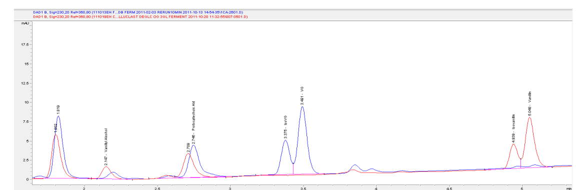


220 UGT enzymes



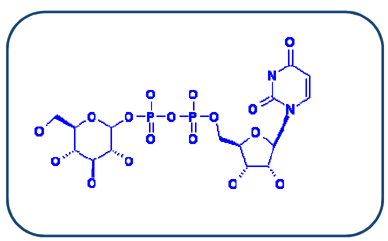


Substrate



Regio-specific glycosylation

detected by HPLC or HPLC-MS



Sugar donor

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The Process of Making an In Cell Glycosylation for a Biosynthetic Process

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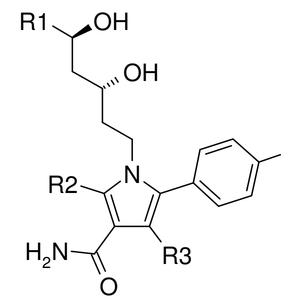

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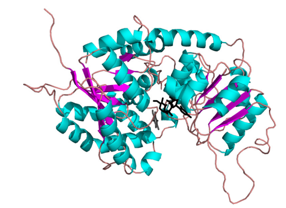

- **3. Testing the glycosylation in the desired *in vivo* system**

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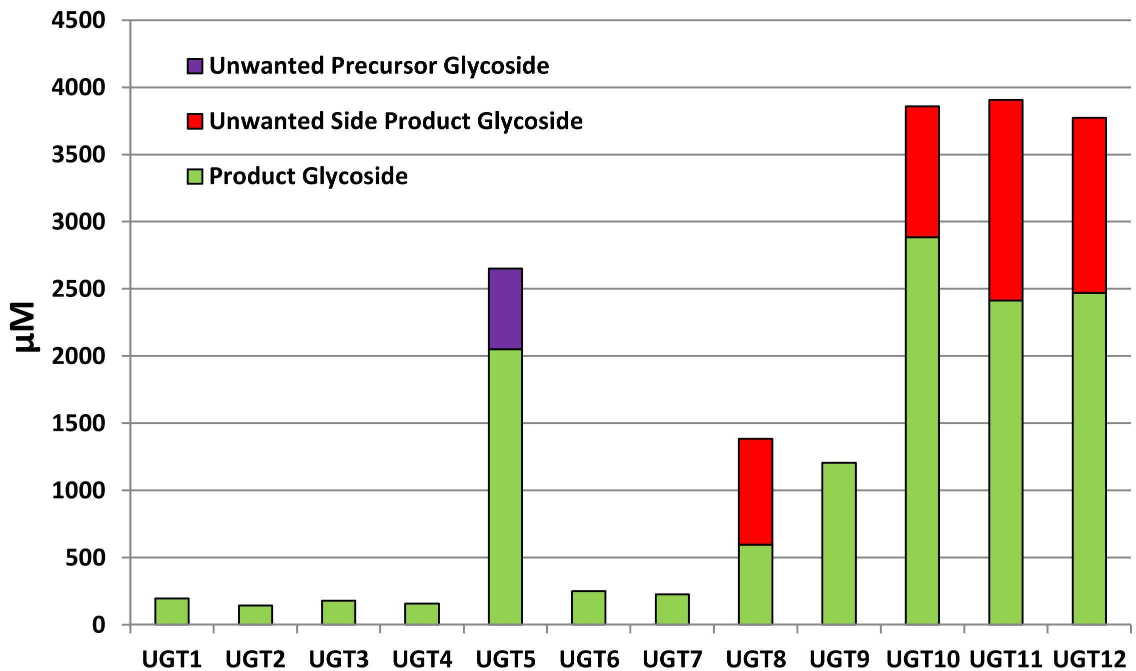
Testing the glycosylation in the Desired *In Vivo* System



- **An example showing varying glycosylation of heterologous pathway intermediates and end product *in vivo***
  - Though a number of UGTs are found to do the reaction *in vitro* they may perform quite differently *in vivo*

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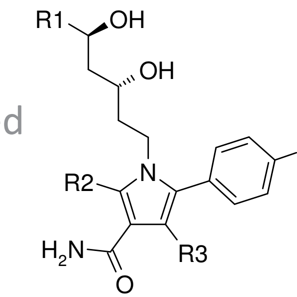
The Process of Making an In Cell Glycosylation for a Biosynthetic Process

- **1. Prerequisites:**
  - Product needs to have a suitable hydroxy, thio or amino group that can be glycosylated
  - The added sugar moiety is to some extent “wasted”. Product therefore needs to be a high or medium priced product
    - Not suitable for biofuel production



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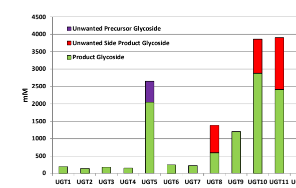
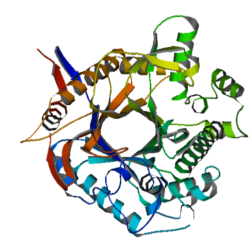
- **3. Testing the glycosylation in the desired in vivo system**



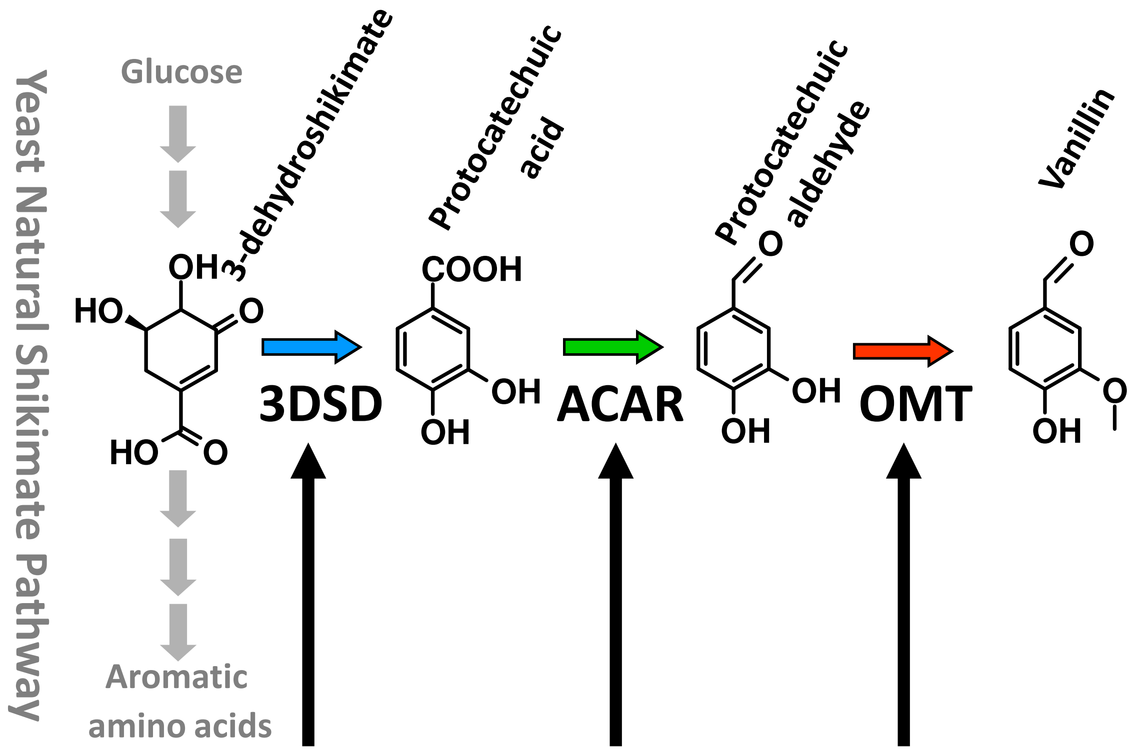
- **4. Find a suitable method for deglycosylation, to release the final product (if necessary)**
  - Hydrolysis of glycosidic bonds can both be done chemically or enzymatically using β-glycosidases

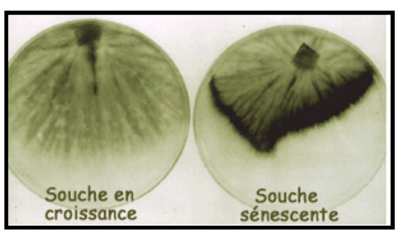


Vanillin Case

Biosynthesis in Yeast

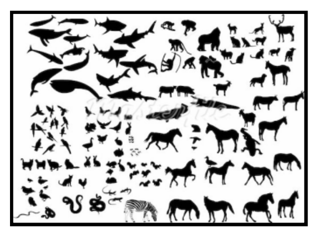
De-novo Vanillin Biosynthesis Pathway





Fungus
(Podospora anserina)

Bacterium
(Nocardia sp.)

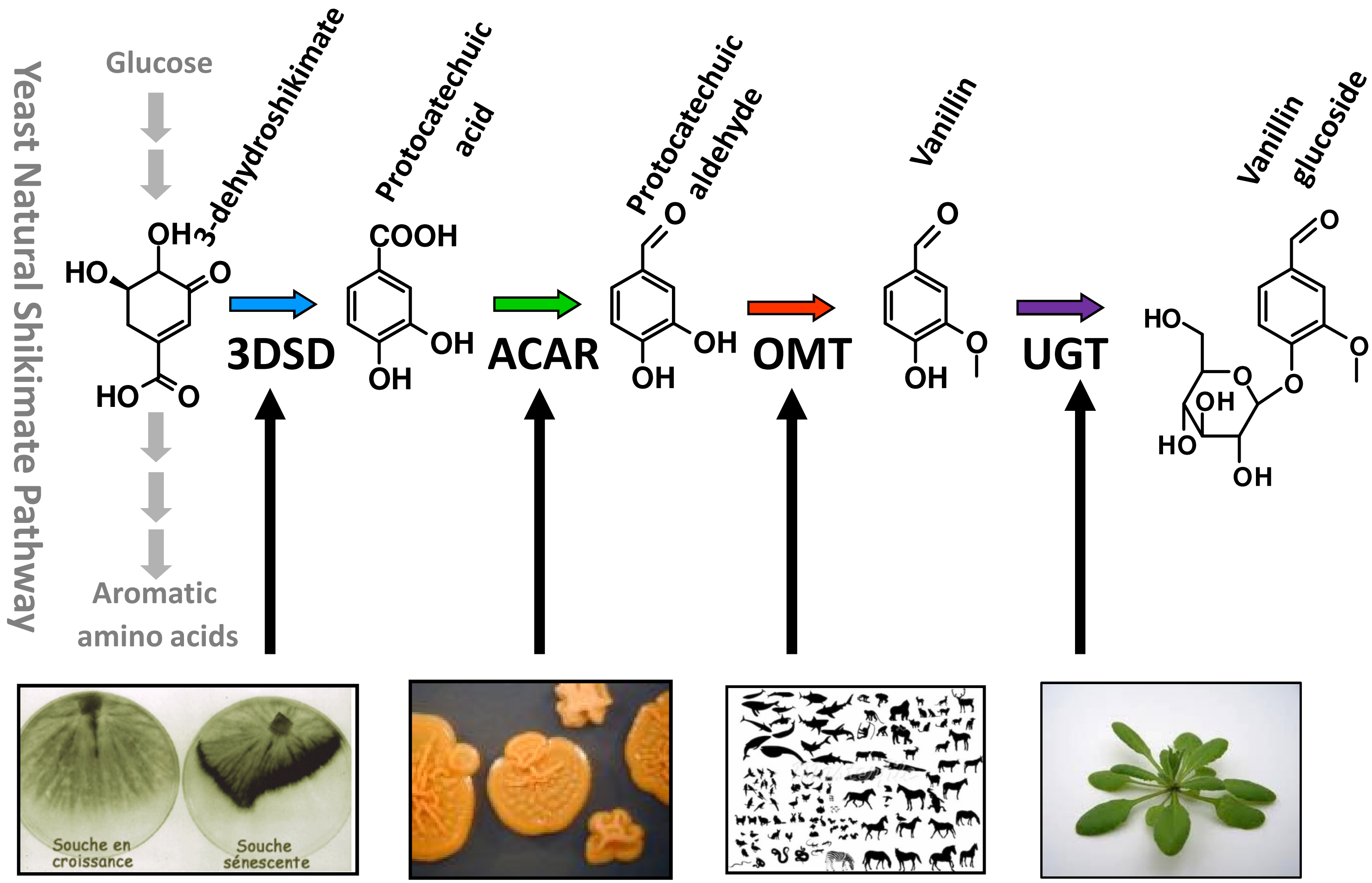


Mammalian
Synthetic

- **De-novo vanillin pathway assembled in *Saccharomyces cerevisisae* from genes of fungal, mammal and bacterial origin**
- **Main issue with microbial production of vanillin is the toxicity**
  - Negatively affects growth of *Saccharomyces cerevisiae* even at 0.5 g/L

(Hansen et al., 2009 AEM)

Glycosylation of Vanillin Product to Solve Toxicity Issue?



Fungus
(Podospora anserina)

Bacterium
(Nocardia sp.)

Mammalian
Synthetic

Plant
(Arabidopsis thaliana)

(Hansen et al., 2009 AEM)

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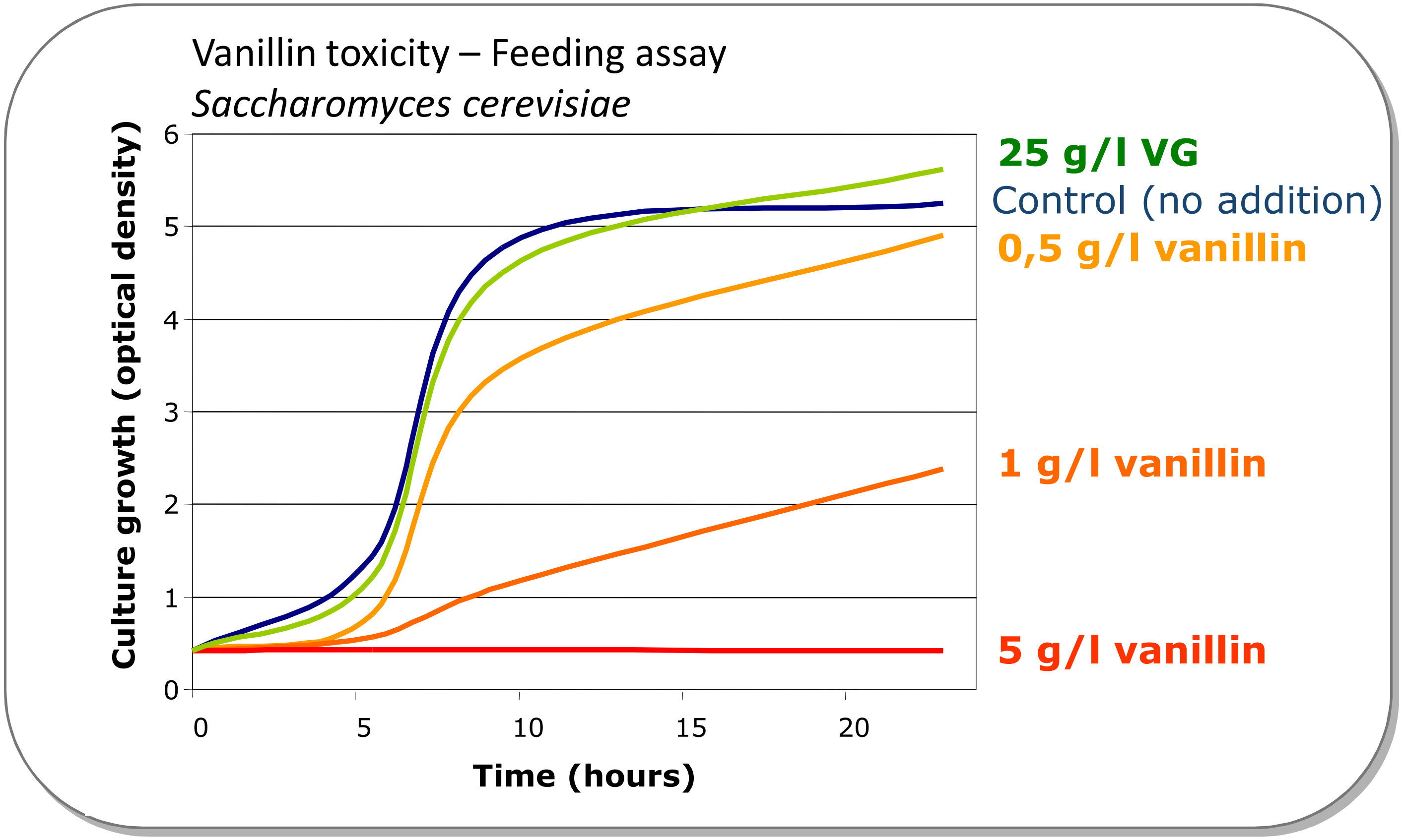
Toxicity of Vanillin vs. Vanillin Glucoside

- **Glycosylation of vanillin solves toxicity issue**
  - **Vanillin toxic even at 0.5 g/l**
  - **Vanillin glucoside non-toxic even at 25 g/l**
  - **Thus VG is at least 50 times less toxic than vanillin**



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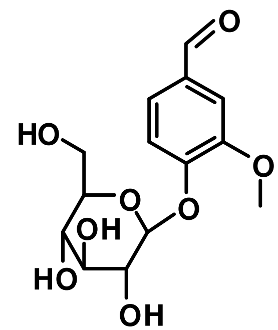
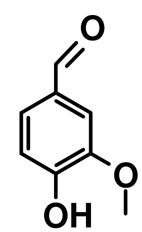
Solubility of Vanillin vs. Vanillin Glucoside

- **Water solubility of vanillin and VG depends on ionization, pH and temperature and different values have been reported**



Vanillin glucoside

- **Predicted solubility of vanillin glucoside in water 25°C is 351 mM (110.5 g/L)**
  - Our experiments showed 114 mM

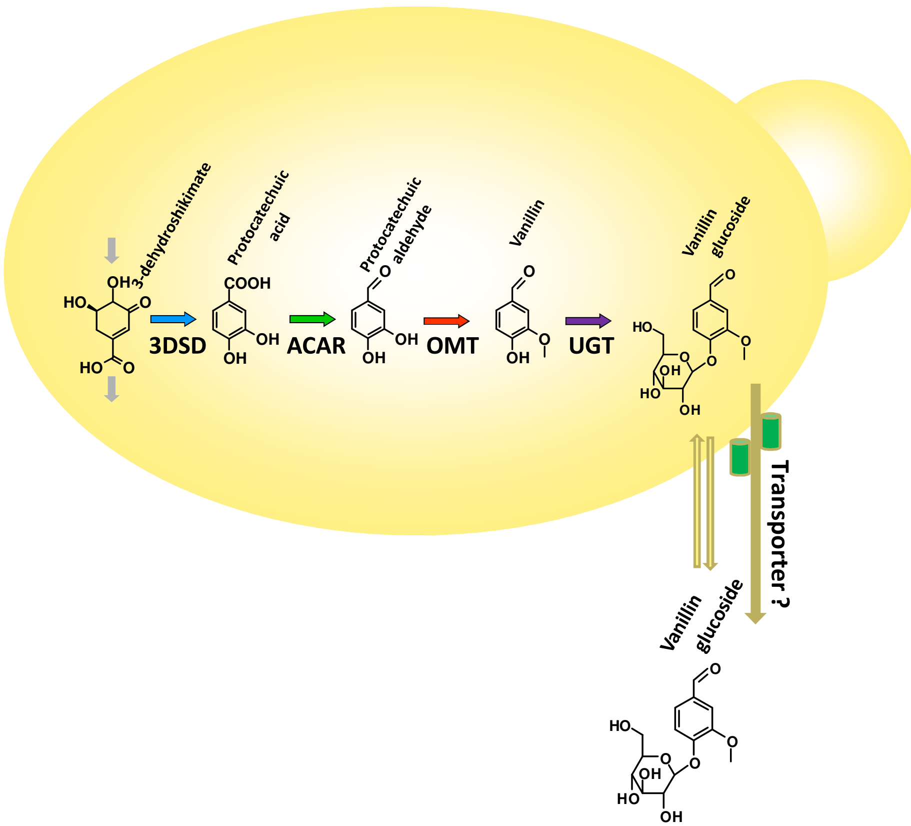


Vanillin

- **Predicted solubility of vanillin is 45 mM (6.875 g/L)**
  - Our experiments showed 41mM

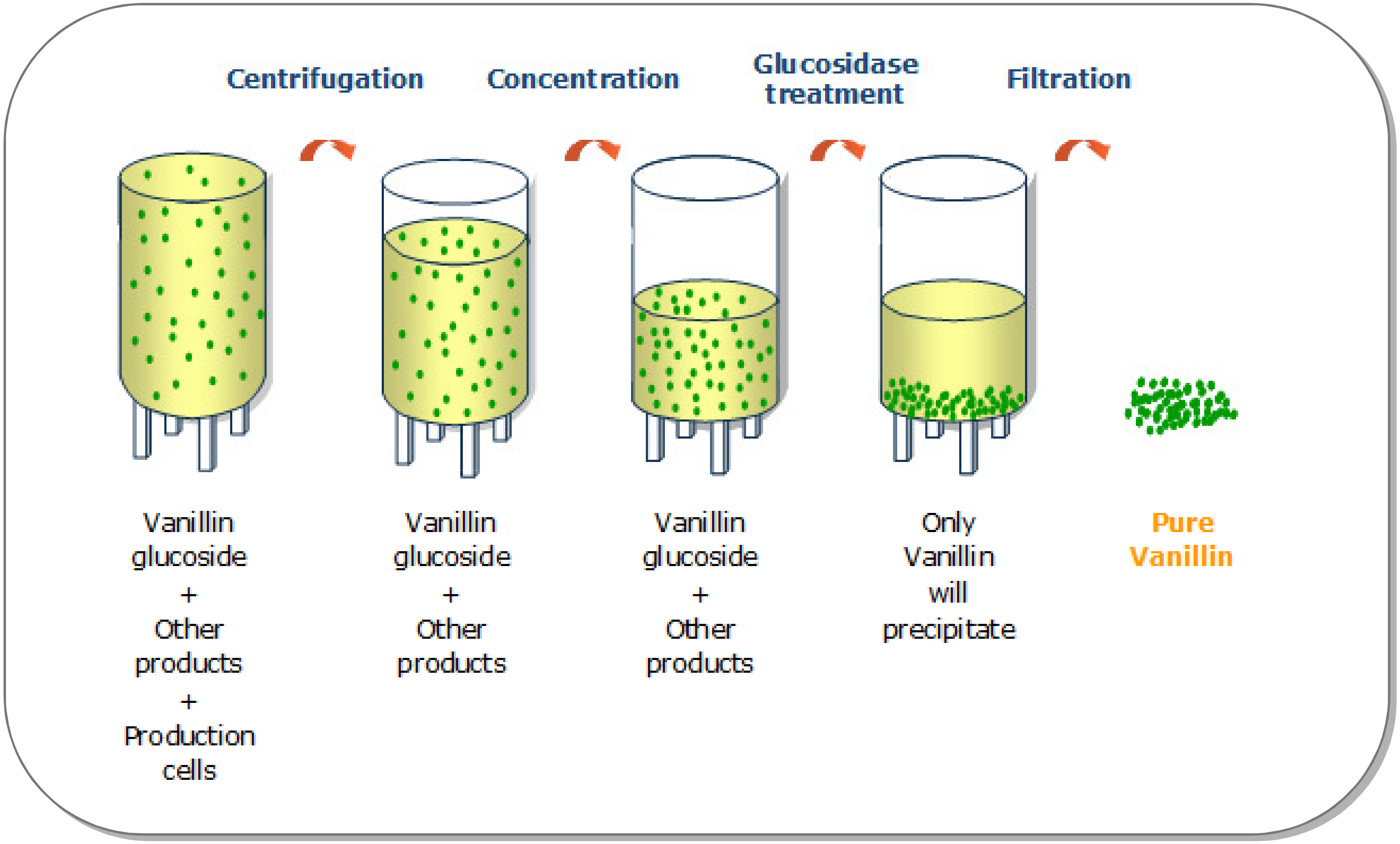
- **Gain in solubility thus in the 2-8 fold range**

Creating a Metabolic Sink in the Vanillin Pathway



- **Vanillin glucoside is readily excreted from the cell**
  - The glycosylation step removes the vanillin and thereby helps to create sink conditions that provides extra “pull” in the pathway
  - Concentration ratio Intracellular:extracellular is approx. 1:5, which indicates that active excretion is involved

Purification of Vanillin via Deglucosylation



- Vanillin glucoside produced in yeast gets excreted efficiently
- Differential solubility of vanillin and vanillin glucoside means vanillin can be purified after glucosidase treatment

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Towards Getting Fermentation Derived Vanillin on Market

- **The use of glycosylation technology has allowed for a high vanillin glucoside titer**
  - Production cost is now at a level where fermentation derived vanillin is competitive in certain markets

- **2011 targets for vanilla programme was achieved**
  - Scale-up scheduled to be initiated early 2013

Saffron Case

Biosynthesis in yeast

Saffron

- **The characteristic saffron flavour, colour and odour come from several components**
  - Most important are picrocrocin, crocin and safranal – all present in the crocus stigma

Crocus sativus

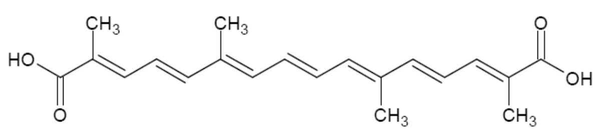
- **The colour of saffron is primarily the result of α-crocin (*trans*-crocetin di-(β-D-gentiobiosyl) ester)**

- **Principal aroma constituent is safranal, which is released from picrocrocin**

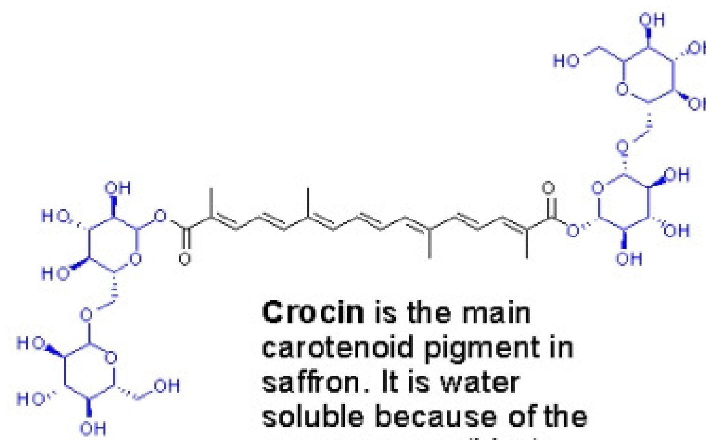
Picrocrocin

Safranal gives saffror it's characteristic aron

- **Purpose of the project is to produce the key compounds picrocrocin and crocin in yeast via fermentation**
  - Crocin is a tetra glucosylated crocetin molecule
  - Picrocrocin is a mono glucoside



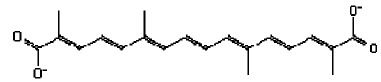
Crocetin (The aglycon of crocin)



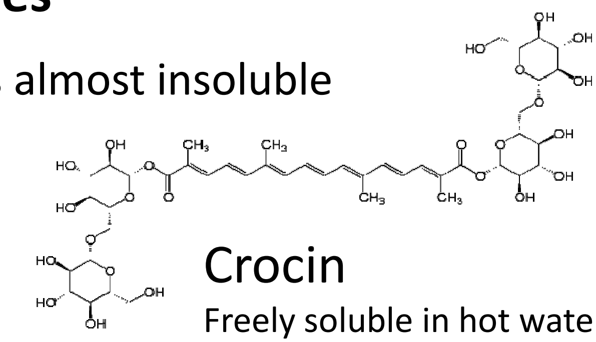
Crocin is the main carotenoid pigment in saffron. It is water soluble because of the sugar groups (blue)

Saffron

- **The end products are glycosides themselves**
- **Advantages of producing a glycoside still applies**
  - Solubility: Crocin is water soluble, while crocetin is almost insoluble



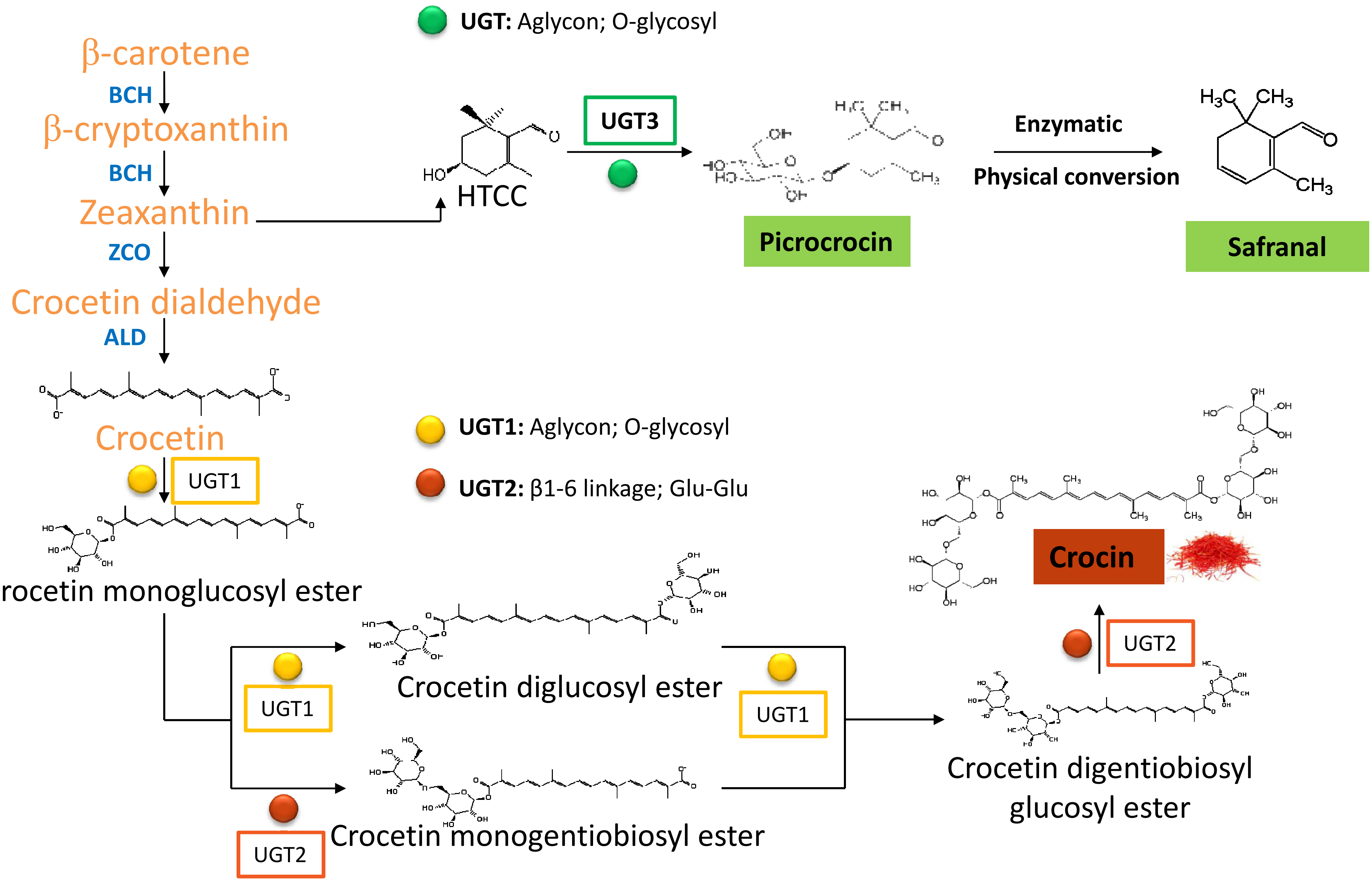
Crocetin
Sparingly soluble in water
(Merck index)



Crocin
Freely soluble in hot water
(Merck index)

- **Main advantages of producing key saffron components by fermentation:**
  - Allows “saffron” to be available at a much decreased price, which will both expand existing markets and open new ones
  - Eliminate the many complexities involved in the current supply chain
  - By making each of the key components separately it will enable the production of customised forms that are for example particularly rich in aroma, taste or colour and that can be adapted to specific food formulations and regional preferences

Saffron Pathways



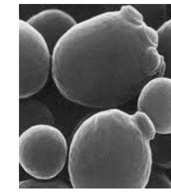
Stevia

Biosynthesis in yeast

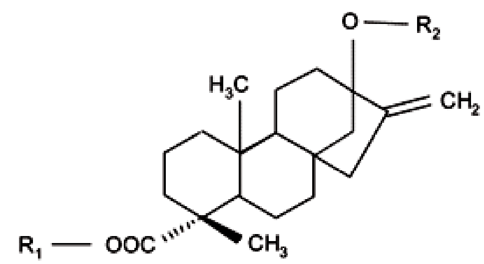
Stevia – Production of Rebaudiosides in Yeast

- **Steviol glycosides originating from the plant *Stevia rebaudiana* are widely used as natural high intensity sweeteners**

- **Functional pathway established in bakers yeast** (*Sacchromyces cerevisiae*)



- **Taste pattern of the stevia glycosides is modulated by the glycosylation pattern**
  - Difference in sweetness, bitterness and liqurice aftertaste



Stevia rebaudiana

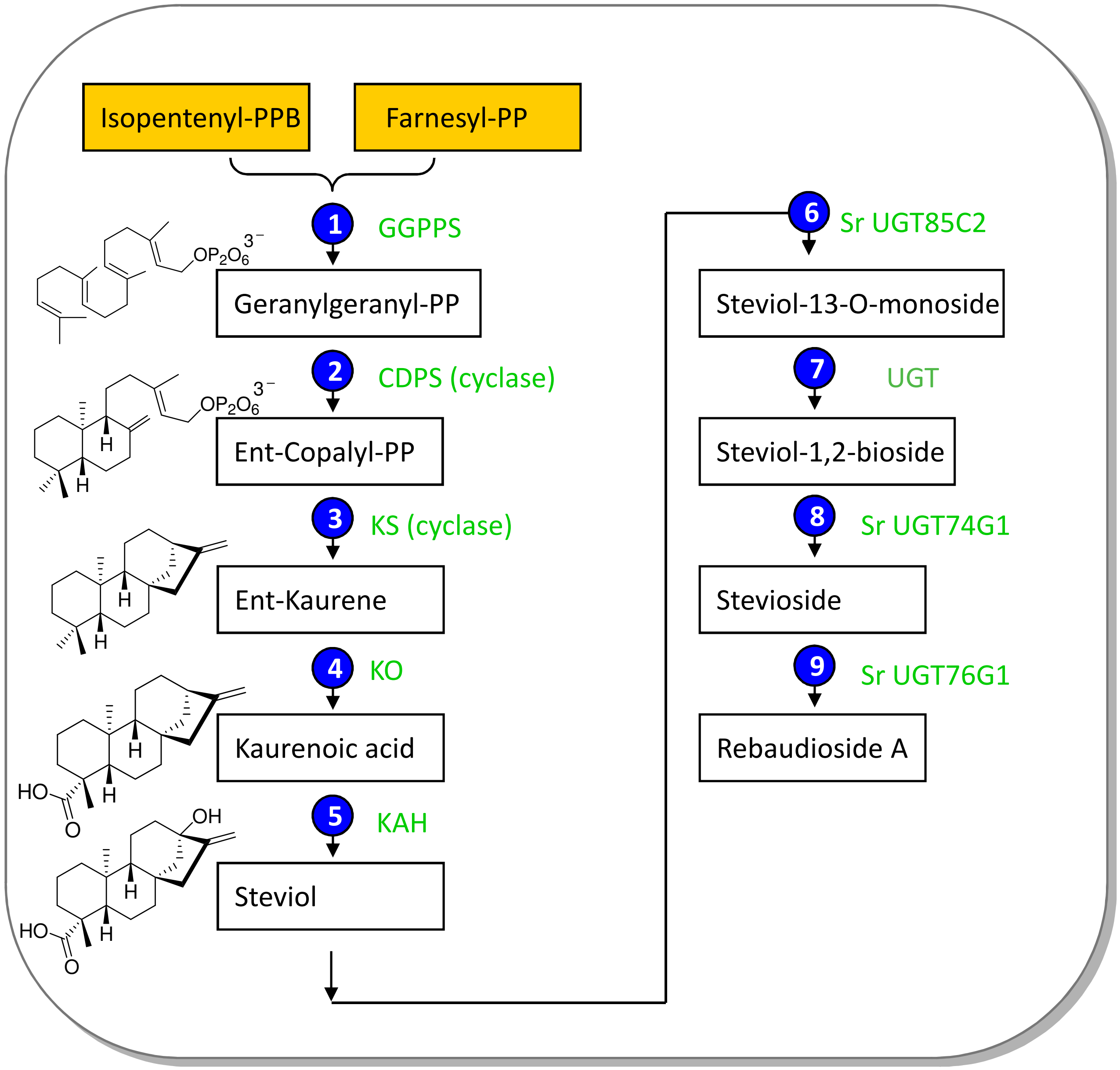
Deterpene glycoside	R_1[a]	R_2[a]	Sweening potency (sucrose = 1)
Steviolbioside	H	glc^{2}—^{1}glc	100 – 125
Rubusoside	glc	glc	100 – 120
Stevioside	glc	glc^{2}—^{1}glc	150 – 300
Rebaudioside A	glc	glc$_3$ ^{2}—^{1}glc, ^{1}glc	250 – 450
Rebaudioside B	H	glc$_3$ ^{2}—^{1}glc, ^{1}glc	300 – 350
Rebaudioside C (dulcoside B)	glc	glc$_3$ ^{2}—^{1}rhm, ^{1}glc	500 – 120
Rebaudioside D	glc ^{2}—^{1} glc	glc$_3$ ^{2}—^{1}glc, ^{1}glc	250 – 450
Rebaudioside E	glc ^{2}—^{1} glc	glc ^{2}—^{1} glc	150 – 300
Dulcoside A	glc	glc ^{2}—^{1} rham	50 – 120

[a] glc, β-D-glucopyranosyl; rham,α-L-rhamnopyranosyl.

Figure 1: Structure of some stevia glycosides (Crammer and Ikan, 1987).

The Rebaudioside A Biosynthetic Pathway

- **Naturally occuring glycosylation pathway in *Stevia rebaudiana***
- **Glycosylation pathway functional in yeast**
- **From glucose to rebaudiosides**
  - Precursor pathways
    - 5-7 steps to prenyl phosphates
  - Steviol pathway
    - 5 steps to steviol
  - Glycosylation pathway
    - 4 steps to RebA, 5 to RebD



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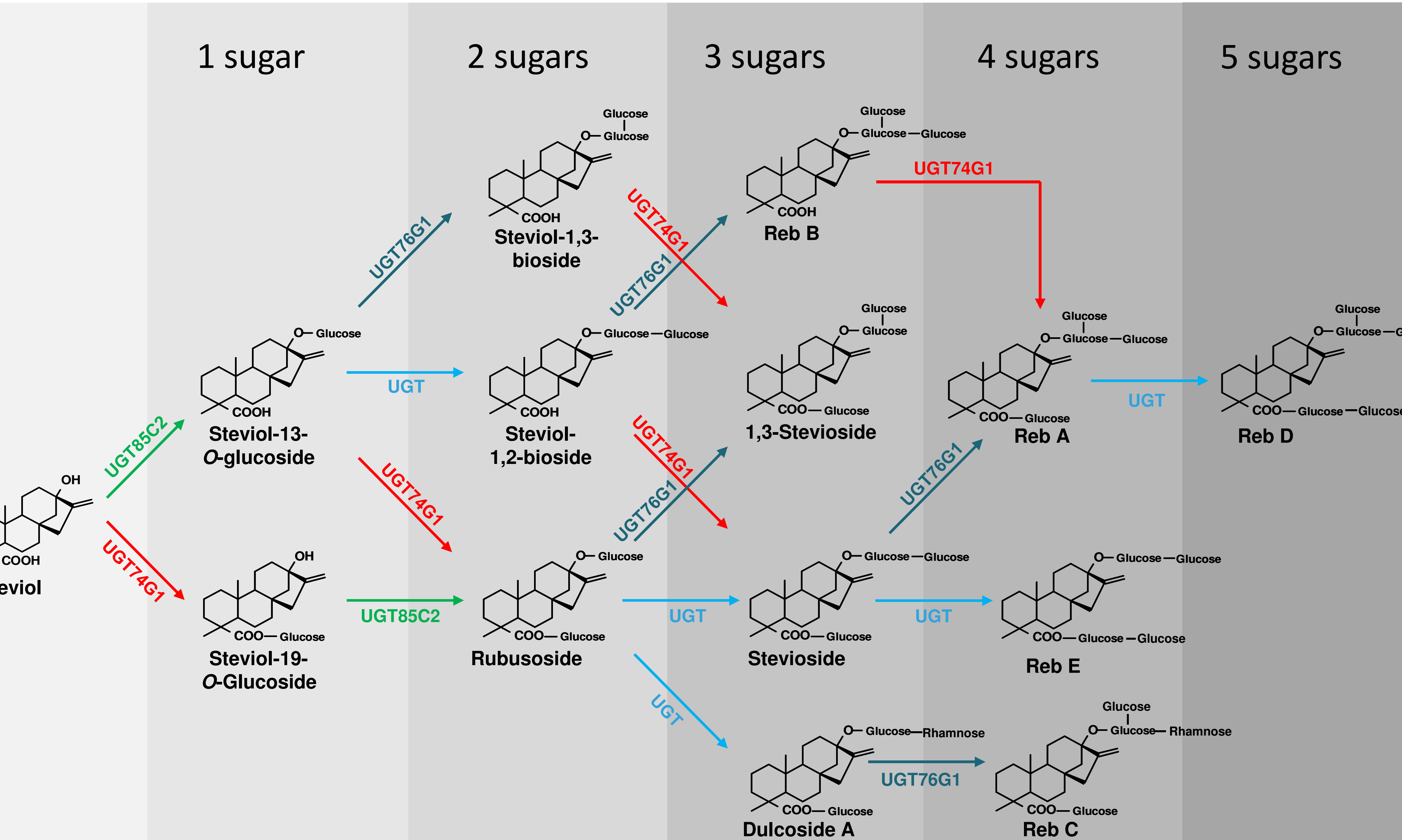
Overview of the Main Steviol Glycosylations



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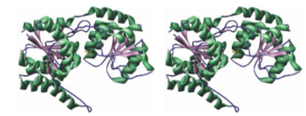
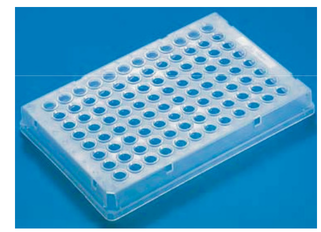
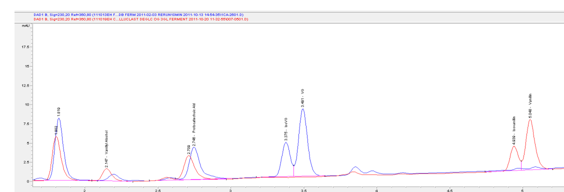
Stevia by Fermentation

- **Production by fermentation allows pure Stevia sweetener components to be produced**
  - Several of the most attractive Stevia components are present at very low concentrations in the plant and thus very hard to extract
  - Glycosylation platform allows for production of specific steviosides/rebaudiosides by selection and use of regio-specific UGTs







Regio-specific glycosylation
detected by HPLC or HPLC-MS

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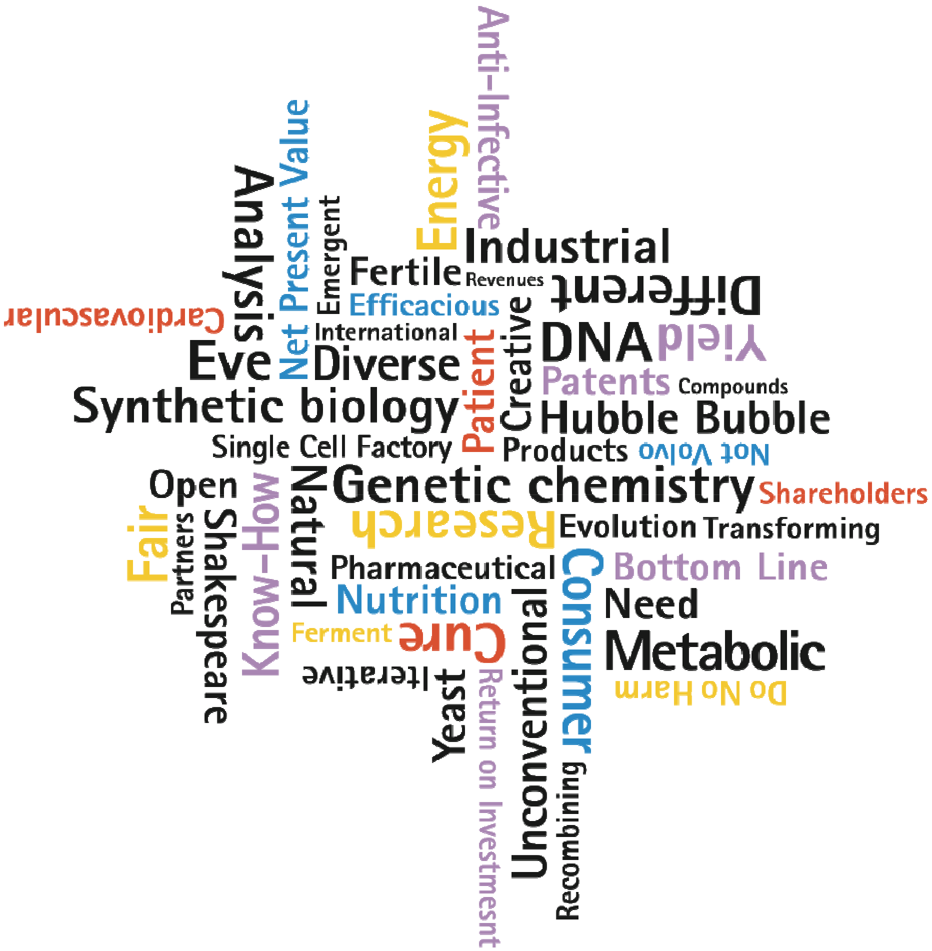
Conclusions

- **Glycosylation is a very valuable tool for improving productivity of heterologous biosynthesis pathways in micro-organisms**
  - Main benefits are:
    - Improved solubility
    - Decreased toxicity
    - Improved “sink” conditions in pathway
- **Several high value products are glycosides**
  - Exact glycosylation patterns crucial for product profile



Thank You

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